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PRINCIPAL INVESTIGATOR: Ruth Lupu, Ph.D.

CONTRACTING ORGANIZATION: University of California at Berkeley
Berkeley, California 94720

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13. ABSTRACT (Maximum 200 Words) Tamoxifen is a common antiestrogen used for the treatment of numerous breast cancer patients, as well as a chemopreventive treatment for the high-risk population. Many breast tumors that initially respond to antiestrogens progress spontaneously to non-responsive tumors, becoming a life-threatening metastatic disease. Contrary to the antiestrogen resistant breast carcinomas, patients who do not develop axillary node metastasis have a good prognosis. Nonetheless, there is still an appreciable relapse rate with some benefit from adjuvant treatments for node-negative disease. However, many women are being treated to benefit those relative few destined to relapse. Consequently, it is important to define the prognostic markers that are involved in breast cancer progression and improve our predictions regarding the group of patients that will benefit from any given treatments. We demonstrated that Cyr61, a protein implicated in angiogenesis is involved in breast cancer progression. Cyr61 is overexpressed in cell lines that do not express estrogen receptor. To demonstrate that Cyr61 is involved in breast cancer, we assessed the extent to which Cyr61 was expressed in tumor specimens. We demonstrated that 30% of invasive breast carcinomas express high levels of Cyr61. It is our hypothesis that Cyr61 expression predicts the need for adjuvant therapy, relapse, and response to antiestrogens. Thus, we will define the role of Cyr61 in invasive versus intraductal breast carcinomas, and determine its prognostic value in node-negative breast cancer patients. This information may also allow us to make use of Cyr61 expression as a marker for improved prediction of particularized therapeutic decisions.				
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EXPRESSION AND SIGNIFICANCE OF CYR61 EXPRESSION IN BREAST CANCER TUMOR SPECIMENS

INTRODUCTION

Breast cancer is a devastating disease that will affect an estimated one in eight women in an average lifespan. Of those women diagnosed with a primary breast cancer, up to 40% will develop metastatic disease. While we are yet unable to cure metastatic disease with current modalities, the antiestrogen Tamoxifen has been shown to produce long-term remissions in these patients. It is a well-tolerated medication, and may in fact have beneficial effects on bone density and reduction of cardiovascular risk factors. Unfortunately, all patients eventually fail tamoxifen therapy by developing resistance to the drug. The response rate to tamoxifen in the metastatic setting varies from 50-75% in estrogen receptor positive (ER+) tumors. Although the presence of ER is employed to predict the hormone dependency of a tumor, the relationship with response to endocrine therapy is not absolute (not all the ER+ tumors respond to endocrine therapy).

Significant levels of estrogen receptor (ER) have been detected in more than 60% of human breast cancers, but at best only two-thirds of these ER+ tumors respond to endocrine therapy. Why this should occur is still unclear. We have previously demonstrated that Cyr61, an angiogenic factor, is involved in breast cancer progression and the acquisition of antiestrogen resistance. More importantly, we showed that Cyr61 is expressed in 30% of breast carcinomas, all of which are estrogen receptor-negative (ER-). These experimental studies have demonstrated a relationship between expression of Cyr61, estrogen independence, and tamoxifen-resistance.

The line of reasoning of our proposal is based upon the function of Cyr61 in distinguishing between those tumors that will be sensitive to or resistant to Tamoxifen, and in distinguishing between those patients that will benefit from adjuvant therapy. There are several independent aspects to this reasoning: First, there is the evidence that Tamoxifen administration to ANN breast cancer patients induces a substantial regression of the tumor, as well as increasing disease-free survival. Second, invasive breast carcinomas often are estrogen-independent and antiestrogen resistant. Lastly, Cyr61 expression correlates with lack of estrogen receptor expression, hormone independence and antiestrogen resistance.

HYPOTHESIS:

Cyr61 expression in breast biopsies associates clinically with HRG, ER and PgR and may predict response to anti-E2 and need of adjuvant therapy.

RATIONALE:

Prediction of breast cancer recurrence can be assessed based on the size of the tumor, number of lymph nodes involved, ER and PgR status, and histological grade. It is known that adjuvant therapy improves long-term survival in breast cancer patients. The present therapeutic goal is to identify appropriate adjuvant regimens for different groups of patients, to reduce the toxicity and improve the efficacy of the drugs. Axillary node-negative (ANN) breast cancer patients have a good prognosis, however there is still an appreciable relapse rate. Adjuvant treatments will benefit some patients with ANN disease. However, large numbers of women (including those in whom no recurrence will occur) must be treated to benefit those destined to relapse. It is important to improve our predictions regarding which group of patients will benefit from these treatments.

A critical point of our proposal resides also in the function of Cyr61 in determining sensitivity vs. resistance of breast cancer to Tamoxifen (Tam). The administration of Tam to patients with ANN breast cancer induces a substantial regression of the tumor and an increase in disease-free survival. These data will clarify the variable responses of breast cancer patients to Tam and elucidate the role of Cyr61 in breast cancer. This information will be invaluable in understanding resistance/sensitivity to Tam, and might also allow us to use them as markers for a better prediction of therapeutic decisions. The data will be correlated with prognostic markers and clinical history. Specifically, we will assess whether expression of Cyr61 correlates with stage, grade, HRG, erbB-2 ER, and PgR status, disease-free survival, metastasis, relapse, and overall survival.

AIMS:

To examine expression of Cyr61 in histological subtypes of breast cancer tumors: To determine expression of Cyr61, we will use an anti-Cyr61 antibody (Ab) for immunohistochemistry (IHC). The detection of Cyr61 in breast cancer will provide a predictive/prognostic tool. This information could be of significance in intraductal, versus invasive, breast cancer and in lymph node-negative vs. positive patients. We will determine the significance of Cyr61 expression in two main groups of specimens:

- 1) One group is composed of ANN subjects, in which we wish to clarify the expression of Cyr61 as determinant of the possible consequent relapse.
- 2) The second group is composed of random specimens, representing different subtypes of in situ and invasive breast cancer: in this case our goal is to define the possible role of Cyr61 in regulating the pace of the tumor and the passage from an initial stage to a more advanced one. The immunohistochemistry results will be reviewed by Dr. Lloveras, a breast cancer pathologist. Stained sections will be reviewed double blind, labeled only with a code from the source of the tumor banks. Once the pathologists have reviewed the cases, the code will be broken, allowing the identification and the release of relevant clinical information and the information will be statistically analyzed.
 - For understanding the prognostic value of Cyr61 expression in node-negative patients and to determine the role of Cyr61 in identifying those patients likely to recur—We will use a study (>400 specimens) in collaboration with Dr. Andrullis.
 - In defining the overall role of Cyr61 expression in invasive vs. intraductal tumors—These analyses will examine the joint variation of Cyr61 and HRG. This study will be performed in collaboration with Dr. Colomer. We have already obtained several hundred samples, and we will study about 400 specimens.

RESULTS:

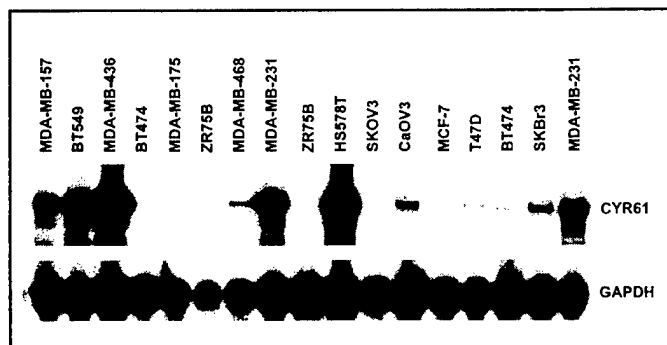
1: Cyr61 (mRNA and protein) is overexpressed in HRG-positive and ER-negative breast cancer cells.

We first wanted to assess the expression of Cyr61 and the ability of an anti-Cyr61 antibody to stained paraffin embedded sections. This was accomplished using a number of breast cancer cells by RNase protection and Western blot analyses. Since the murine Cyr61 expression was regulated by serum, to ensure accurate measurements of Cyr61 expression, we cultured the cell lines under serum-depleted conditions, otherwise stated. Using RNA protection assays, the levels of Cyr61 were measured in RNA isolated from breast cancer cells. Since Cyr61 is a secreted protein, its expression was measured in conditioned media derived from breast cancer cells by Western blot analysis using an anti-Cyr61 polyclonal antibody (kindly provided by Dr. Lester Lau, at UIC). As can be seen in Figures 1A and 1B, and summarized on Table 1, Cyr61 is expressed in many breast cancer cells at the mRNA and protein levels.

Our studies demonstrate that Cyr61 overexpression correlates with HRG overexpression and with lack of ER expression, and correlates with the aggressive phenotype of the breast cancer cells, *i.e.*, their ability to invade, and to metastasize *in vitro* and *in vivo*. Of note, in serum containing media the T47D mRNA and the adjacent sample of BT474 mRNA, express higher levels of Cyr61 as compared with the same cell lines in the absence of serum (fourth lane from left). From this observation, it would appear that Cyr61 is not only regulated by serum (as shown in the mouse model) but it is possible that another component in the serum, besides E2, induces its regulation. Given the redundancy apparent for many cellular functions, some cells may not require Cyr61 to perform its functions, or may be selective in the Cyr61 functions they require. We have recently shown that Cyr61 is regulated by E2 in breast cancer cells.

Figure 1 A-B: Cyr61 is expressed in ER-negative and metastatic breast cancer cells.

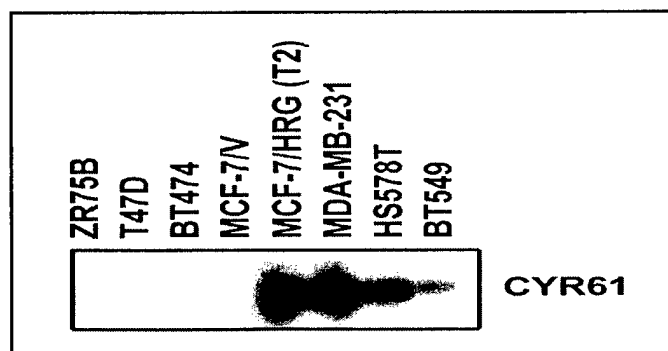
A)



Cells were cultured in IMEM containing 5% fetal bovine serum (FBS). Maintaining the cells in this manner does not pre select for populations later in the study. The medium was replaced by serum-free-medium (otherwise stated in the results) during a four-day period, to ensure

complete depletion of serum for four days. Total RNA was isolated for analysis by RNase protection assay. Protection assays were performed using 40 µg of total RNA isolated from the different breast cancer cells. The loading was monitored by GAPDH as control.

B)



Cells were maintained as described in A. When cells reached about 80% confluence, conditioned medium (CM) was harvested and heparin chromatography was performed. Elutions were performed with increasing concentrations of NaCl and

fractions were collected. Protein concentration of fractions was determined for all the fractions at high salt elution (0.9M NaCl), and Western blot analysis was performed using an anti-Cyr61 antibody.

Table 1: Cyr61 and HRG are co-expressed, and overexpression correlates with E2-independence, antiestrogen-resistance and aggressiveness.

Cell line	Cyr61	HRG	ER	Vimentin	Invasive in vitro	Metastatic <i>in vivo</i>
MCF-7	-	-	++++	-	- ¹	-
T47D	-	-	++	-	- ¹	-
BT474	-	-	++	-	- ¹	-
MDA-MB-175	-	+/- ³	+	-	- ¹	-
ZR75B	-	+/- ³	+	-	- ¹	-
MDA-MB-468	+	-	-	-	+ ²	-
SKBR-3	+	-	-	-	+ ²	-
MDA-MB157	++	++	-	++	+	ND
MDA-MB-436	+++	+++	-	+++	+++	ND
BT-549	+++	+++	-	+++	+++	+
MDA-MB-231	++++	++++	-	+++	++++	+
HS578T	++++	++++	-	+++	++++	+

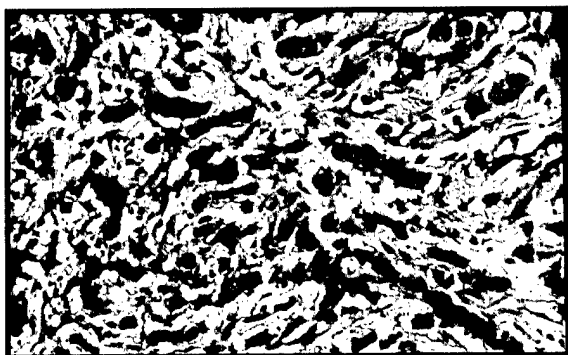
¹Require E2 for invasion *in vitro* and growth *in vivo*, never metastasize *in vivo*. ²Require ligand (EGF or HRG respectively) to invade, never metastasize *in vivo*. ³E2 induces the expression of HRG. ND: Not determined

2. Cyr61 is expressed in 30% of breast cancer tumor biopsies:

To determine whether the Cyr61 expression observed in our cellular models might have broader relevance, we investigated Cyr61 expression in human breast cancer biopsies. We considered these results a more logical next step than further cell line studies, such as transfection, since those studies are time-consuming and would be rendered largely irrelevant for breast cancer if we could not detect Cyr61 expression in the human disease. We initially examined 10 breast tumor biopsies by Western blot analysis, detecting strong immunoreactivity in 4 out of 10 samples (Figure 2B). Recently, we developed an immunohistochemical assay (IHC) for the detection of Cyr61 in paraffin-embedded sections. Our preliminary IHC analyses, performed on only a small number of cases (n=55), demonstrate that Cyr61 is expressed in 30% of invasive breast carcinoma, while it does not appear to be expressed in normal human breast tissue (Figure 2A).

Figure 2A-B: Cyr61 expression is detected in 30% of breast cancer biopsies.

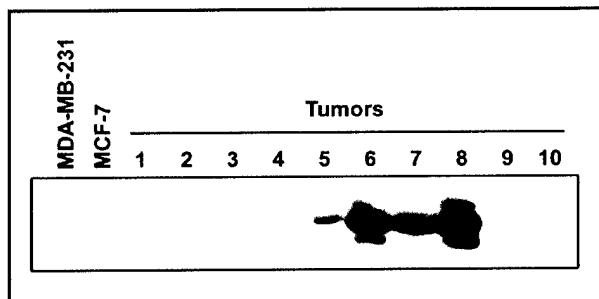
A.



Expression and localization of Cyr61 is detected, by IHC staining, in paraffin-embedded specimens, obtained from a breast carcinoma. The multistep detection system, Avidin-Biotin-Peroxidase method, was used. The tissue was deparaffined and rehydrated with xylene and alcohol. After a 30 min. treatment with blocking solution (goat

serum 10% in PBS), an anti-Cyr61 antibody was applied for 1 hour (5 µg/ml).

B.



Tumors were lysed and equal amounts of protein were loaded on a 4-20% SDS-PAGE. Blots were visualized using an anti-Cyr61 antibody.

3. Expression of Cyr61 in Invasive Breast Carcinomas: In defining the overall role of Cyr61 expression in invasive vs. intraductal tumors. *These analyses will examine the joint variation of Cyr61 and HRG.* We have already stained one hundred samples for Cyr61 expression, and we will study in total more than 300 specimens. Correlations with known prognostic markers such as stage of progression, heregulin (HRG) and ER expression was also determined. We tested the expression of Cyr61 in invasive breast carcinomas and determined that it is expressed in 28% of breast carcinomas and it is co-expressed with HRG and inversely correlated with ER expression as shown in Table 2.

Table 2: Cyr61 and HRG are co-expressed in invasive breast carcinomas (n100)

	Cyr61	HRG	ER
Cyr61	28%	32%	3%
HRG	32%	30%	4%
ER	3%	4%	3%

CONCLUSIONS:

These data provide considerable evidence that Cyr61 may play an important role in breast cancer. However, the number of samples tested was too low to propose its predictive and/or prognostic relevance. No additional clinical information was available on these specimens; thus, additional studies are necessary to establish the magnitude and the impact of Cyr61 expression in breast cancer. Our previous data reveal that Cyr6 expression represents a critical factor in invasive breast carcinomas, and this is consistent with the idea that expression may be an important event in carcinogenesis. MCF-7 cells exhibit an early phenotype *in vivo* (noninvasive, non-metastatic, antiestrogen and hormone-responsive), because these cells are derived from a metastatic lesion, they express low to undetectable levels of Cyr61. The cell culture and the human biopsy data are consistent with Cyr61 playing an important role in HRG induction of breast cancer progression from an E2-dependent to an E2-independent and antiestrogen resistant phenotype. We have determined that Cyr61 expression correlates with HRG expression and inversely correlates with ER status. The expression of Cyr61 is extremely significant and will provide an additional tool into breast cancer diagnosis and prognosis.

FUTURE TASKS:

1. Examine the expression of Cyr61 (protein and mRNA) in different histological subtypes of breast tumors: Our first goal is to determine the significance of Cyr61 expression in two main groups of specimens. The first group is composed of lymph node-negative specimens, in which we wish to clarify the expression of Cyr61 as the determinant of the possible consequent relapse. The second group is composed of random specimens, representing the different subtypes of *in situ* and invasive breast carcinoma. In this second case, our goal is to define the possible role of HRG in regulating the pace of the tumor growth and the passage from an initial stage to a more advanced one. To determine expression of Cyr61, we will use an anti-Cyr61 antibody (for immunohistochemistry) and Biotinilated-Cyr61-riboprobes (for *in situ* hybridization).

- ***We will continue defining the overall role of Cyr61 expression in invasive vs. intraductal tumors***—These analyses has been started and will be completed in the next six months. Correlations with known prognostic markers such as stage of progression and ER/HRG status will be determined.
- ***We will begin to address the prognostic value of Cyr61 expression in node-negative patients and to determine the role of Cyr61 in identifying those patients likely to recur***—Correlations with known prognostic markers such as stage of progression and ER/HRG status will be determined.

Stained sections will be reviewed double blind, labeled only with a code from the source of the tumor banks. Once the pathologists have reviewed the cases, the code will be broken, allowing the identification and the release of relevant clinical information. The information will be statistically analyzed. These tumor specimens have already been or are currently being evaluated for HRG expression.

2. Prognostic and Diagnostic Significance of HRG expression: The statistical analyses will be described with respect to the two primary objectives:

- Comparing Cyr61 expression in invasive vs. intraductal tumors: Expression of Cyr61 by immunohistochemistry will be both represented as a four level ordinal variable, *i.e.*, "-", "+", "++", "+++", and collapsed into a binary variable representing low (-/+) vs. high (++/+++) expression. Chi-square tests of trend and association will be used to compare Cyr61 expression between invasive and intraductal tumors (40). Logistic regression (with binary Cyr61 expression as the dependent variable) will be used to determine whether the association between histology and Cyr61 expression is confounded by or modified by other patient or tumor factors such as age, race, *erbB-2*, ER, PgR status, and HRG (41).
- Correlating the joint expression of Cyr61 with prognosis, and response to Tam: Expression of Cyr61 will be correlated with recurrence-free and overall survival among the following patient groups: node-positive and -negative patients (combined and separately), Tam treated patients, and Adriamycin treated patients. Within these broad patient groupings, Kaplan-Meier estimates of survival probability will be compared among patients with different levels of Cyr61 expression using both log rank and Gehan-Wilcoxon tests. Cox proportional hazard models will be used to adjust survival comparisons for the presence of other prognostic factors including stage, grade, ER/PR status, ploidy, and S-phase. By incorporating co-variants in the model for these factors, we will determine the independent prognostic value of Cyr61 joint expression. Interactions between Cyr61 and ER status will be investigated by fitting cross-product terms. Stepwise variable selection will be performed manually, based on change in log-likelihood, effect on other parameters in the model, and validity considerations, *i.e.*, retaining marginally significant variables which are biologically meaningful.
- Power Calculations: Power calculations will be shown separately for the two objectives shown above. Comparing Cyr61 expression in invasive vs. intraductal carcinomas. Power calculations are based on the ability to detect differences in the proportion of Cyr61 expressing tumors between the two histological groups. Thus, power is based on simple differences in binomial proportions. We have access to a sample of 300 intraductal tumors, and a much larger sample of invasive tumors. With

300 patients per group, we will have more than 80% power to detect an increase of at least 15 percentage points in Cyr61 expression among invasive tumors. Thus, the study has more than adequate power to detect biologically significant differences in expression for this objective. Expected numbers of deaths and recurrences for relevant subgroup analyses will be given by applying the relevant rates to subgroups in this combined patient sample. We have made the conservative assumption that approximately 50% of node-negative, and 25% of node-positive patients will have been treated with Tam, and approximately 25% of node-positive patients will have been treated with Adriamycin. Thus, we see that for the subgroups of interest in this objective, statistical power will be more than adequate to detect small increases in recurrence risk, treatment failure, and cancer deaths. Even if our estimates of the number of Tam or Adriamycin treated patients are too high, reducing the estimated number of recurrences in these groups by 50% would still leave more than adequate statistical power to detect relative risks of 2.0 or greater. Although not a stated component of this objective, exploration of subgroups within these categories will be possible given the sample sizes shown above.

ANTICIPATED RESULTS FROM THE PROPOSED STUDIES:

The immunohistochemical study is exploratory, but will determine the likely incidence of detectable Cyr61 expression and the range of intensity. The ER/HRG study will determine whether large scale testing is warranted. Our approach should enable us clearly to establish the predictive/prognostic power of Cyr61 expression and any association among Cyr61, HRG, ER, and PgR expression in breast carcinomas.